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Environmental Organisms as Risk Factors in the Occurrence of Mastitis in Dairy Buffaloes with Suggested Methods of Control. A Field Study

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Abstract: Mastitis results in tremendous economic losses to the dairy industry all over the world. A field study was carried out on 200 buffalo cows (native breed) aged from 2 to 4 years old in herd groups near Alexandria Desert Road (El-Khatatba), Giza, Egypt, whereas unhygienic measures were prevailed. 33 out of 200 examined animals were mastitic and the clinically diseased cases with acute mastitis were characterized by the visible moderate swelling and firmness of infected quarters, sign of chunks of milk, milk clots and sometimes viscous milk. Samples were taken from clinical mastitic quarters, bulk milk tank, milking machine and water sources (pipes and tank) and bacteriologically examined to identify the causative agent. It was found that the major causative agents isolated from the clinically mastitic cases were *Escherichia coli* (43.8 %), *Staph aureus* (37.4 %) and *Mycoplasma bovigenitalium* (16.5 %). When applying different lines of treatment, the diseased animals were classified into 3 groups (11 each). The first group received local treatment with intramammary infusion of 125 mg of ceftiofur hydrochloride, while the second group received systemic treatment with I/M injection of both enrofloxacin (5mg/kg body weight for 5 successive days) and I/V injection of carprofen (2.9ml/kg body weight) as an immunomodulator drug. The third group received a combination of both local and systemic treatment. The recovery percentage was 54.5, 80.9 and 90.9 for the three groups, respectively. The highest incidence of recovery was obtained in the third group, in which stimulation of the innate immune mechanisms of the animal was applied. The discard milk from sick or antibiotics treatment cases was examined before and after heat treatment to 55, 60, 65 and 70°C for either 2, 3, 5, 10 and 30 minutes. It could be concluded that both environmental and hygienic measures surrounded the animals constitute a major risk factors in the occurrence of mastitis, so continuous bacteriological investigation together with treatment of both mastitic animals and discard milk must be done.

Key words: Buffaloes *S. aureus* · *E. coli* · *M. bovigenitalium* · Enrofloxacin ceftiofur · Carprofen

INTRODUCTION

The general health and well being of individuals depends largely on meeting basic nutritional needs. Milk and fermented milk products have formed an important part of daily nutrition and the variety of products produced from milk has increased dramatically over the years, as modern food processing technologies have improved [1]. Also, an increase in the global population coupled with the increasing demands for milk as an economic food and as an industrial raw food product has necessitated an increase in production by dairy farms [2].

Mastitis is one of the most significant health problems of dairy herds as it cause physical, chemical and bacteriological changes in the milk of dairy animals resulting in inferior quality and quantity of produced milk [3]. Therefore, public interest in the welfare of animal production recognition of mastitis as a major source of pain and stress for the affected cows and buffaloes give added focus to mastitis as a major problem [4]. Buffalo cows are the main dairy animals in some developing countries worldwide despite this species tends to have relatively slow rate of reproduction and more reproductive problems such as inactive ovaries, long calving intervals and mastitis [5-7]. Clinical mastitis is easily diagnosed in

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buffaloes based on apparent signs and symptoms and palpation of udder [8]. Economic losses were summarized by Varshney and Naresh [9] and Getahun *et al*. [10] as loss in milk production, discarded abnormal milk and treated milk with antibiotics, degrading milk quality and price (due to high bacterial or somatic cell count), cost of drugs, veterinary services, increased labor costs, increased risk of subsequent mastitis, herd replacement and problems related to antibiotics residues in milk and its products. Globally, the losses due to mastitis amount to about 53 billon dollars annually [2].

Improving udder health and decreasing the incidence of udder infection and inflammation in dairy herds, will result in increased milk production as huge losses are directly or indirectly incurred through loss of milk during treatment periods, culling of cows and death of clinically infected cattle [11]. Mastitis control programmes addressing various aspects of dairy farming such as feeding practices, animal husbandry, hygiene and general health care can contribute towards reducing the incidence of udder infections. Treating infection with antimicrobials can, in conjunction with good farming practices, assist in this endeavor to eliminate, or at least decrease, the incidence of mastitis infection within a dairy herd [11].

Clinical mastitis has been reported to be caused by a variety of factors, including contaminated dry-off preparation [12], teat wipes [13] and wash water used to clean udders prior to milking [14] and the ability of organism to readily grow in soil and water and its relative resistance to chemical disinfectants [15]. It is characterized by sudden onset, swelling and redness of the udder, pain and reduced and altered milk secretion from the affected quarters. The milk may have clots, flakes or of watery in consistency and accompanied by fever, depression and anorexia.

The main etiological agents responsible for mastitis infections can be divided into different groups of organisms depending on the source of the organism involved. These include contagious pathogens, environmental bacteria, opportunistic bacteria and other organisms that less frequently cause mastitis less frequently [16]. Environmental pathogens are found in the immediate surroundings of the cow, such as the sawdust and bedding of housed cows, the manure of cattle and the soil. Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced [17]. The microorganisms that responsible for most episodes of the environmental mastitis in dairy cows and buffaloes are *Staphylococcus aureus*, *streptococcus spp.* [18], *E. coli (E. coli), Salmonella, Klebsiella, Coryne bacterium* and *Mycoplasma spp.* [19-20]. These are spread from infected to clean udders during the milking process through contaminated milker's hands, cloth towels used to wash or dry udder of more than one animal and possibly by flies [20].

Numerous agents can cause mastitis in dairy cows, but *Staph. aureus* is the most common etiological agent of bovine mastitis [21, 22]. Although various management practices for decreasing the prevalence of *Staph. aureus* have been adopted under modern dairying, many dairies still have some levels of infection with *Staph. aureus* [22, 23].

E. coli occupies the environmental reservoirs likely to result in teat end contamination, where the infection with *E. coli* is typically of rapid onset and acute [24]. Therefore, it is quickly recognized and persists for only short period of time, also its opportunity to transmit a new infection is low, under the correct circumstances, but the ability of the gland to acquire a new infection from other sources is high [25].

Prevalence of mycoplasma mastitis appears to be increased in many locations throughout the world. Twelve species of Mycoplasma and Acheloplasma have been isolated in milk samples from mastitic cases in adult dairy animals, of these mycoplasma, *M. bovigenitalium* was the first to be recognized as it was suggested that calves could be infected by pathogenic mycoplasma through contaminated milk, colostrums, or vaginal secretion at birth or the organism introduced through the teat canal of the adult one or by hematogenous spread to the mammary gland [19, 26].

The determination of milk somatic cell count (SCC) is widely used to monitor udder health and the milk quality. The elevated SCC consists primarily of leucocytes which include macrophages, lymphocytes and neutrophils. During inflammation, major increase in SCC is because of the influx of neutrophils into milk and at this time over 90% of the cells may be polymorphonuclear (PMN) leukocytes [20]. The higher the SCC, the greater is the risk of raw milk contamination with pathogens and antibiotic residues. Furthermore, high SCC raises the suspicion that the raw milk is produced under poor standards of hygiene and from unhealthy animals [27]. Milk from normal uninfected quarters generally contain below 200,000 somatic cells /ml. A value of SCC above 300,000 is considered abnormal whereas it will be an indication of inflammation in the udder [28-30].

According to Harmon [31], the mastitis or elevated SCC is associated with a decrease in lactose, α-lactoalbumin and fat in milk because of reduced synthetic activity in the mammary tissue.

Mastitis control was already an important dairy health management initiative for many years. A5-point mastitis control plan was developed in major extension efforts throughout the dairy industry, The five points listed by Giesecke *et al.* [32] include: (A) Teat disinfection after milking; (B) Proper hygiene and milking procedures and adequate milking equipment; (C) Culling of chronically mastitis cows; (D) Antibiotic dry-cow therapy; (E) Prompt treatment of clinical mastitis during dry period and during lactation. Failure of the 5-point plan to control other groups of bacteria (e.g. *Streptococcus uberis* and the various coliform species) led to the hypothesis that such bacteria were transmitted to the gland from sources other than infected/diseased mammary quarters (i.e."the environment"). Indeed, such was the success of these procedures worldwide that the terms "contagious" and "environmental" pathogen (referring to those organisms that are and are not controlled by the five point plan [25].

The defense mechanism of the animal and udder, including mechanical and immunological, are essential for the outcome of an infection. However, during certain periods the defense is suppressed and the risk for udder infections and mastitis increases. To avoid udder infections and following mastitis, it may be beneficial to find ways to stimulate the animal's immune defense for more efficient resistance against and/or elimination of infection [33]. More interest has been directed towards ways to stimulate the innate immune mechanisms of the animal in general and /or locally in the udder [34]. Non-steroidal anti-inflammatory (NSAID) with antipyretic, analgesic and anti-inflammatory activities, have been used as adjunctive or alternative therapy to systemic or intramammary antibiotics [35].

Insufficient contact of the antibiotics with pathogenic bacteria at the site of infection is a major cause of mastitis treatment failure [36]. The route of administration, intramammary or parenteral, of medicinal products to treat mastitis is an important issue. It determines the biological barriers encountered by the active compound and the routes by which it may make contact with the causal microorganism [37].

Obvious beneficial trends were recorded in the treatment of clinical mastitis using a combination of both local and systematic lines. Treatment decisions for clinical mastitis are generally motivated by a desire to return milk to a saleable state [38]. Cure percentages were not only satisfied, but also accompanied with improvement of milk quality, general behavior and appetite of the herd [39].

Ceftiofur is a new broad-spectrum third generation cephalosporin antibiotics for veterinary use. It inhibits bacterial cell wall synthesis by interfering with enzymes essential for peptidoglycan synthesis [40]. Consequently; this new antibiotic should be effective against a wide variety of mastitis pathogens, including environmental mastitis pathogens [41, 42].

Enrofloxacin is a fluoroquinolone developed exclusively for veterinary use and exhibit high bactericidal activity against a broad spectrum of aerobic Gram-negative, some Gram-positive bacteria and *Mycoplasma* spp [43]. A combination of enrofloxacin and levamisole as an immunomodulators were found to clear 100 % of the infection due to *Strep. agalactiae*, *disgalactiae* and *Micrococcus spp*. [44].

Carprofen is one of the NSAIDS which acts by selective inhibition of the synthesis of a particular class of prostaglandins and endo-peroxidases and inhibition of biochemical reactions, in addition to cyclo-oxygenase (COX) inhibition [45, 46].

Discarded milk from sick and antibiotics treatment cases is often used as an economical alternative to milk replacer at dairy farms in which it poses a health hazard to calves or human if the milk comes from cases with mastitis. So, heat treatment to the milk result in destruction of microorganisms causing mastitis specially mycoplasma [47].

Little work has been carried out on dairy buffaloes reared by local farmers to establish the etiology of increasing prevalence of mastitis and its source of contamination through isolation and identification of the prevailing causal bacterial organisms with suggested lines of treatment for both mastitic animals and their milk.

MATERIALS AND METHODS

Animals: This study was carried out in a farm at Alexandria Desert Road, Egypt in which 33 out of 200 clinically mastitic animals aged from 2 to 4 years old in herd groups were used in the period of study. All animals were housed in a separate free yard on straw bedding floor whereas unhygienic measures were prevailed. They were milked manually twice daily and the milk was collected in milk tank until treatment. They receive their needs of water through a common water trough. All housing and management decisions were the responsibility of the farmer. Buffaloes were assigned randomly to one of three antibiotic treatment groups (n= 11 animals per group).

Physical Examination of the udder. The physical condition of the udder and its evaluation was conducted after complete evacuation of the milk according to Massart-Leen *et al*. [48] and scored as Normal (score 1), the udder was pliable when totally milked out, heat pain, redness and and/or swelling were not detected; animals exhibited no signs of discomfort. Slight swelling (score 2), the udder was less pliable with some firmness, redness, heat and pain were generally not detectable; animals exhibited no signs of discomfort. Moderate swelling (score 3), the udder was definitely firm, reddened and warm in touch; animals generally exhibited signs of discomfort (irritable, performed stepping motion with feet and/or kicked during the preparing and milking procedures). Sever swelling (score 4), the udder was very hard, red, hot and noticeably larger than the other quarters; the animals was extremely uncomfortable and very irritable. Scar tissue (score 5), the udder was generally pliable and a hardened lump was palpable and it could be detected from milking to milking overtime without change in size, no pain, heat, redness, or swelling was associated with this condition. Edema (score 6), the udder was swollen, reddened, hard and often extended forward toward the navel, as well as posteriorly up the rear quarters where the udder attaches to the body.

Physical examination of milk sample. Appearance of foremilk was scored as follows, Normal (score 1), flakes (score 2), small slugs (score 3), large slugs (score 4), stringy and watery (score 5)) and bloody (score 6). The mammary glands were considered to have clinical mastitis when the udder or milk score was 3 or higher.

Sampling. Milk samples were collected using standard procedures described by Harmon *et al.* [49]. Briefly, after discarding the first few milk drops, milk samples were taken from all clinically mastitic cows by wiping the teats with 70 % ethyl alcohol with paying extra attention to teat orifice. Each milk sample was collected in a sterile screw capped bottle; also bulk milk tank sample was taken aseptically in a sterile flask. Both Milk samples and tank milk samples were sent directly to the laboratory with minimum delay for the routine cultural identification. Milk samples were centrifuged at 300 RPM for 15 Minutes to get the sediment.

Sterile cotton swabs removed from a nutrient broth tube were rubbed onto the hands of milkers at different sites then returned back to nutrient broth tube. Another sterile cotton swab moisted with sterile saline was used to swab the milk machine containers.

Under complete aseptic condition, approximately 100 gram of the soil and bedding materials were collected from the place in which the udder of the recumbent animal was resting. All samples were kept on melting ice (1°C) during transport and sent directly to the laboratory with a minimum of delay for routine culture techniques.

Bacteriological examination. Loopfuls from the milk sediment, bulk milk tank, milker's hands, milk machine containers and the bedding materials were inoculated into a nutrient broth, brain heart infusion broth, then incubated aerobically at 37°C for for 24-48 hours for enhancement of bacterial growth. Subcultures were streaked on 10 % sheep blood agar (for Isolation of *Staph auris*) and macConkey agar plates (for Isolation of *E. coli*) according to Carter and Cole [50]. Suspected colonies were identified on the basis of their cultural, morphological characteristics and biochemical reactions [51-53]. For *Mycoplasma*, loopful was cultured on modified Hayflicks media, incubated at 37°C under 10 % CO2 for 7 – 10 days [54] to find the fried egg appearance of the characteristics' *Mycoplasma* colonies.

Somatic cell count. Linear somatic cell count scoring method was applied on the data of somatic cell count to assess the milk loss [55] in all treated groups.

The treatment trials. All diseased animals were classified into 3 groups (11 animals each). The first group received local treatment by intramammary infusion with 125 mg of ceftiofur hydrochloride (Pfizer Animal Health, Egypt). The second group received systematic treatment by I /M injection of 5mg/kg body weight enrofloxacin and I / V injection of 2.9 ml/100 kg body weight carprofen (Pfizer Animal Health, Egypt). The third group received a combination of both local and systematic treatment. The treatment applied once daily and for 5 successive days. Clinical cure was defined as the disappearance of clinical signs which were observed on day before treatment, on other words, by the return to normal feed intake, good general condition, absence of udder edema, normal milk appearance and normal milk yield.

Milk Treatment: *Thermal heat treatment to discard milk*. The discarded milk sample was obtained from mastitic animals (Positive samples for *Staph aureus*, *E.coli* and *Mycoplasma bovigenitalium*) and divided into 5 ml. and stored immediately at 4°C until used (not more than 24 hours). The milk samples were incubated in water bath at 37°C to 20 minutes to stimulate the temperature of the milk on the farm and then the milk was tested to the sensitivity of the isolated microorganisms to 55°, 60, 65 and 70°C for either 2, 3, 5, 10 and 30 minutes. We had also a control negative milk sample that was free from infection. Once, the tested milk samples removed from the heated water bath, it was immediately placed in a room temperature water bath. After stabilization of all samples

to a room temperature, 0.1 ml. of each was cultured to a 0.9 ml. sterile saline and repeat the culture procedures for isolation and identification of the causative microorganisms. The test was conducted according to Butler [56].

RESULTS AND DISCUSSION

There was high prevalence of mastitis in dairy buffalocows in our field conditions, which ultimately reflects the bad quality of milk available to the consumers [57]. Clinically mastitic cases was detected by clinically infected quarters often showing moderate swelling, firmness, visible signs of chunks of milk, clots in milk and in some cases, the milk become viscous. Mastitis generally results from interaction between a variety of microbial infections and host responses in the udder and it is influenced by management practices [58]. Factors which predispose to mastitis include mostly environmental aspects such as poor hygiene, poor husbandry, overcrowding, bad ventilation, poor milking technique and malfunction of milking machines [58]. The environmental pathogens are most often responsible for the clinical cases in which about 50% of environmental pathogens display clinical symptoms and nearly 60 to 70% of them exist for less than 30 days and are not easily detected. 33 out of 200 examined native buffaloes were diseased.

Samples were positive for several bacterial organisms as present in Tables 1, 2 in which the most dominating bacterial species were *E. coli*, *staph aureus* and *Mycoplasma bovigenitalium* in a single or mixed infection. The isolated strains were present in high levels in the housed animal's environment, especially in bedding materials and water tank as they act as primary reservoir for these environmental pathogens [59, 60]. The remarkable increase is may be due to passage of milk through the milking equipment which gets contaminated from the polluted water during rinsing with cold water. *Staph. aureus* infections may be of environmental origin as it was detected on many environmental samples [39]. Also, the worth mentioned that the most predominant bacterial isolates were *E. coli* from all the examined samples in which it constitutes a great threat to the consumer in case of inefficient pasteurization since it causes cases of gastroenteritis [52]. The isolation of other bacteria in mixed infection with mycoplasma organisms suggests that this *mycoplasma* may increase the susceptibility of the mammary gland to other pathogens and environmental microorganisms [19]. Therefore, the spreading of acute form of clinical mastitis among animals is a warning message to organize control programs for mastitis. Additionally, many investigations had assured that bacteriological culture is the gold standard method for identifying the intra-mammary infection (IMI) and routine milk cultures should be an

Table 1: Different types of bacterial isolates in clinically mastitic animals.

	Numbers of mastitic cases	%	Rates	Single / Mixed infection
E. coli	17	43.8%	1	Single
			4	Mixed with *Staph aureus*
			3	Mixed with *Mycoplasma.*
Staph aureus	11	37.4%	5	Single
			4	Mixed with *E. coli*
			2	Mixed with *Mycoplasma*
M.bovigenitalium	5	16.5%	2	Mixed with *Staph aureus.*
			3	Mixed with *E. coli*

Table 2: Bacteriological examination of different environmental samples

Samples	*E. coli*	*Staph aureus*	*Mycoplasma bovigenitalium*
Bulk milk	+	+	-
Quarter milk	+	+	+
Water pipes	+	-	-
Water tank	+	-	-
Bedding	+	+	+
Milker's hands	+	-	+
Milking machine	-	-	-

+ means positive results of isolation

- means negative results of isolation

Table 3: The local and systemic lines of treatment with the cure percentages

Different Lines of treatment	Number of tested animals	Cured animals	% of cured animals
First group (local intra-mammary infusion with ceftiofur)	11	6	54.5%
Second group (I/M injection of enrofloxacin and I/V injection of carprofen)	11	9	81.8%
Third group (both lines of treatment)	11	10	90.9%

Table 4: Somatic cell count of the treated grouped

Animal group	Somatic cell count cells / ml.
Normal milk samples	96,000
Clinically mastitic animals	339,000
Local treated groups	225,000
Systemic treated groups	187,000
Local and systemic treatment groups	152,000

ongoing part of any mastitis control program [61]. The result was in accordance with those reported by Sargeant *et al*. [62] and Gonzalo *et al.* [63] who reported that *Staph aureus*, *E. coli* are the most common etiological agents involved in subclinical and clinical reasons of mastitis. They also confirmed that mastitis considered as a multi-factorial disease whereas development of IMI depends on presence of mastitis pathogens (environmental bacteria) and series of additional factors (bad habitat and lack of hygiene) that act concomitantly [58].

Local treatment may be sufficient to induce only 54.5 % cure; the systemic treatment cure % was 81.8, while the application of both lines of treatment had the best cure percentage (90.9 %, Table 3). All systemic reactions disappeared after the completion of the course treatment and the appetite of the animals had increased together with the improvement of the somatic cell count. Our first line of treatment was coincide with that of Oliver *et al*. [42] who found that ceftiofur therapy was effecting in eliminating naturally occurring subclinical IMI in lactating dairy cows caused by several different mastitis pathogens and that extended ceftiofur therapy significantly enhanced treatment efficiency.

Improvement of local clinical signs of the swelling and milk appearance at the level of affected quarters was noticed after intramuscular administration of enrofloxacin and intravenous administration of carprofen. The high cure percentages (81.8 %) may be due to the high bio-availability and high tissue concentration exceeding minimum inhibitory concentration (MIC) values for pathogens [64]. The present treatment schedule is in agreement of Akhtar *et al*. [65] who used enrofloxacin and 3-D Vet for treatment of clinical mastitis, the differences is that in the present study, carprofen was used as anti-inflammatory and immune-potentiator instead of Diclofenace sodium (3-D Vet). It is concluded that carprofen used as immune-modulator to increase the functional capabilities of neutrophils, macrophages and plasma cells. It also, increases the phagocytic and bactericidal activity of neutrophils at the mammary glands, inhibits the biochemical reactions of the most bacterial pathogens and shortens the severity of mastitis [45, 46].

Combination of local and systemic treatment of mastitic cases by antibiotics coupled with immunomodulator was found to be highly efficacious against e nvironmental pathogens causing mastitis. These results are in accordance with those reported by Grewal *et al*. [66] who found that combination of systemic and intramammary infusion is more effective in the treatment of clinical mastitis in buffaloes when cure rate in terms of quarter is considered.

Treatment programme was efficient on both bacteriological cure and SCC of infected quarters in which the somatic cell count in the treated groups was significantly lower than that of the infected group [67], (Table 4). This rise of SCC in the infected groups was attributed to the presence of infection in some quarters of the buffaloes of these groups. This is in line with the findings of Sheldrake *et al.* [68], who also found that a higher elevation in SCC is an indication of inflammation in the udder.

Heat treatment of milk results in destruction of the isolated microorganisms from the mastitic cases which can save produced money on milk replacer to feed calves and also to eliminate the transmission of the organisms [56]. So pasteurization equipments should be available and efficient to inactivate different pathogenic microorganisms.

CONCLUSION AND RECOMMENDATION

E. coli, *Staph aureus* and *Mycoplasma* are the main environmental pathogens that cause severe mastitis in dairy animals, so an effective control system must be applied to prevent or minimize the exposure and the transmission of these pathogens. The animal's environment should be as clean and dry as possible with no access to manure, mud and pools of stagnant water, also calving area must be clean. Post milking teat dipping

with a germicidal dip is recommended. Animals should receive diets which are supplemented with vitamin E and selenium or immunomodulators to reduce incidence of mastitis caused by environmental pathogens. Combination of local and systemic treatment of mastitic cases by antibiotics together with immunomodulators was found to be highly efficacious against environmental pathogens causing mastitis.

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